

Use of Calcein as a Fluorescent Marker for Elasmobranch Vertebral Cartilage

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Abstract.—The effectiveness of calcein as a fluorescent marker of elasmobranch vertebrae was examined in the nurse shark *Ginglymostoma cirratum*. Intramuscular injection of calcein at a standard dosage of 25 mg/kg body weight (BW) produced distinct fluorescent marks in nurse shark vertebrae; however, it also induced physiological stress and mortality. Lower dosages of 10 and 5 mg/kg BW also produced chemical marks suitable for age validation but were not associated with animal death. These data suggest that calcein dosage levels recommended for teleost age validation may be toxic to elasmobranchs. Future studies should focus on the toxicity of lower-dosage calcein injections.

Most studies on elasmobranch age and growth typically use calcified growth zones, visible in vertebral centra, as aging indices (Cailliet 1990). Despite widespread use, several problems have been associated with the analysis of these growth zones (Cailliet and Tanaka 1990). The temporal periodicities associated with growth zone production appear to be variable (Pratt and Casey 1983, Natanson and Cailliet 1990, Branstetter and Musick 1994), thus reinforcing the need for proper age validation. Unfortunately, age validation has been performed in few elasmobranch growth studies (Cailliet 1990, Kusher et al. 1992, Natanson 1993) and remains a high-priority research need.

The validation of age estimates derived from calcified structures is frequently accomplished by using chemical markers. Once administered, these chemicals are deposited at sites of active calcification and produce fluorescent or pigmented marks in calcified structures. The location of these marks can then be examined to determine if the structure

in question accurately records age. A variety of chemical markers including oxytetracycline [OTC] (Yamada 1971; Hettler 1984), calcein (2,4-bis-[N,N'-di(carbomethyl)-aminomethyl] fluorescein; Yamada 1971; Wilson et al. 1987; Hales and Hurley 1991; Monaghan 1993; Brooks et al. 1994; Bumgardner and King 1996), calcein blue (Brooks et al. 1994), and alizarine complexone (Lang and Buxton 1993; Ahrenholz et al. 1994) have proven effective in validating age estimates derived from teleost otoliths. At the time of this study, only OTC had been used to validate growth zone periodicity in elasmobranch vertebrae (Holden and Vince 1973; Smith 1984; Branstetter 1987; Brown and Gruber 1988; Tanaka 1990; Kusher et al. 1992; Natanson 1993). No published studies have examined the effectiveness of alternative chemical markers in elasmobranch age validation, yet such studies may prove useful in the improvement of elasmobranch aging biotechnology.

The goal of this study was to evaluate the effectiveness of calcein as a chemical marker for elasmobranch age validation. Since this study began, one group has reported the effectiveness of calcein-injection in age validation of gummy shark *Mustelus antarcticus* and school shark (tope) *Galeorhinus galeus* (Walker et al. 1995). The current study describes the ability of calcein to produce fluorescent marks in vertebral cartilage of nurse shark *Ginglymostoma cirratum*.

Methods

Five nurse sharks (age-0 to age-1) were collected in the Florida Keys and transported to the laboratory, where they were maintained in outdoor circular tanks (capacity, 3,200–12,000 L). The experimental tanks operated as either open or recirculating systems and were subjected to natural

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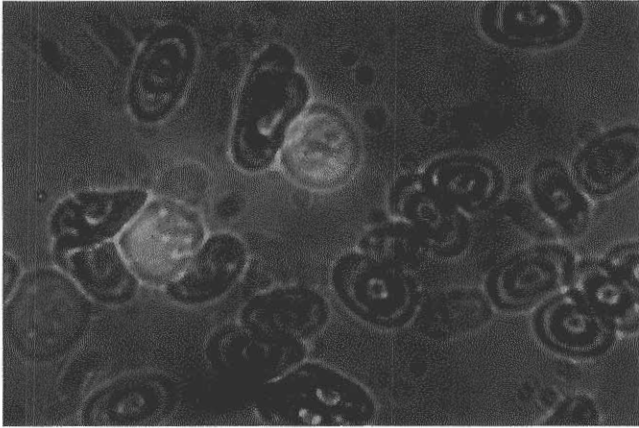


FIGURE 1.—Phagocytosis of calcein by a granulocyte in the serum of a nurse shark injected with 25 mg calcein/kg body weight (blue light illumination).

photoperiod and temperature regimes. After an acclimation period of 2 weeks, all animals were sexed, measured (stretched total length), weighed, and tagged with rototags for individual identification. Afterwards, tagged nurse sharks were injected in the lateral musculature with an elasmobranch Ringer's solution vehicle (Forster et al. 1972) containing calcein at a dosage of 25 mg/kg body weight (BW). After receiving the injections, all nurse sharks were returned to experimental tanks for captive maintenance. Nurse sharks were fed to satiation three times per week with Atlantic threadfins *Polydactylus oligodon* or Spanish sardine *Sardinella aurita*.

Four of the five specimens died 12, 13, 16, and 17 d, respectively, after chemical injection. Nec-

ropsies were performed on these specimens, and tissue samples from the liver and injection-site musculature were collected for histologic examination. Tissue samples were fixed in 70% ethanol, sectioned on a cryostat freezing microtome, and stained with hematoxylin and eosin. Vertebral centra were obtained from all specimens for localization of chemical markers. Centra were cleared of connective tissue, fixed in 70% ethanol for 24 h, and air-dried. Whole and longitudinally sectioned centra were examined under blue (470 nm) light to detect calcein marks. Tissue and vertebral samples were also obtained and processed for the remaining specimen (CAL-25), which was anesthetized with tricaine methanesulfonate (MS-222; 1 g/L) and sacrificed 7 months after injection.



FIGURE 2.—Fluorescent calcein marker in longitudinally sectioned vertebral cartilage from a nurse shark injected with 25 mg calcein/kg body weight (blue light illumination).

Because of the unanticipated deaths of most of the experimental animals, another goal of this study was to determine the effectiveness of low-dosage calcein injections. Two additional nurse sharks (CAL-10 and CAL-5) were injected with calcein at dosage levels of 10 and 5 mg/kg BW, respectively. After 12 months, these specimens were anesthetized and sacrificed, and vertebral samples were processed to detect calcein fluorescence.

Results and Discussion

Mortality was high (80%) for nurse sharks injected with calcein at 25 mg/kg BW. One or more of the following symptoms preceded death in these individuals: "arching" posture of the cephalic region, erratic swimming behavior, and heavy active gilling. Externally, all specimens had widespread orange pigmentation of the skin, buccopharyngeal cavity, and teeth. Under ultraviolet light (254 nm), it was determined that calcein had been deposited within the calcified structure of several dermal denticles and peripherally on gill filaments. No external lesions or parasitic infections were apparent on any specimens. Internally, a discrete area of tissue located near the mandibular angle appeared bright yellow in at least two specimens. One specimen also had an unusually enlarged gall bladder, hemorrhaging of the spleen, and bright yellow fluid in the coelomic cavity and intestinal lumen. Impression smears obtained from liver and spleen samples of the dead specimens demonstrated that calcein was present in the liver but was absent from the spleen. Histological tissue sections did not indicate any signs of hepatocyte damage or muscular necrosis. Examination of body fluids revealed the presence of calcein in serum and urine, both of which fluoresced bright yellow-green when exposed to blue and ultraviolet light. In addition, microscopic examination of blood smears indicated that calcein was being phagocytized by granulocytes (Figure 1). High concentrations of granulocytes were also observed in histologic liver sections.

Except for orange skin pigmentation, no external or internal abnormalities were observed in specimens CAL-25, CAL-10, or CAL-5. All three nurse sharks appeared to be healthy and grew in length and weight during the experimental period.

Calcein at dosages as low as 5 mg/kg BW produced distinct fluorescent marks in nurse shark vertebrae. All calcein marks appeared bright yellow-green under blue light illumination (Figure 2) and were sufficient to facilitate age validation. Un-

like OTC marks, repeated illumination of calcein marks did not reduce their clarity or brightness.

The results of this study indicate that calcein, at dosages as low as 5 mg/kg BW, is an effective chemical marker for elasmobranch age validation. Unlike OTC, benefits of calcein injection include the production of distinct, long-lasting vertebral markers that can be seen easily with harmless blue light. Higher dosage levels previously recommended for teleost studies (Monaghan 1993), however, are toxic to nurse sharks and, thus, greatly limit the effectiveness of this technique. Injection of calcein at 25 mg/kg caused rapid mortality in four of five captive nurse sharks, all of which displayed behavior consistent with physiological stress (Cliff and Thurman 1984). Despite this observation, the underlying cause of mortality in these sharks is unclear. Observations of heavy active gilling and the presence of calcein on gill filaments suggested that calcein might have affected respiration in experimental nurse sharks, but this suggestion is still conjectural and more supporting evidence is required. Although Walker et al. (1995) did not associate mortality in gummy shark and school shark with calcein injection, those researchers also observed negative effects (tissue irritation) with this technique (R. Officer, Victorian Fisheries Research Institute, Australia, personal communication).

The health and survival of shark specimen CAL-5 and the production of distinct fluorescent marks by calcein injected at 5 mg/kg BW strongly support the use of lower dosage levels. Other studies have also observed the effectiveness of low-dosage calcein injections (Monaghan 1993), and Yamada (1971) suggested that the concentration of calcein required to produce a suitable mark was one-quarter that of OTC. Further studies on the dose-related toxicity of calcein in elasmobranchs need to be completed before its use is recommended as a vertebral marker. Once these studies are completed, calcein may provide an appropriate alternative to OTC use. In addition, calcein may eventually be used in conjunction with OTC to facilitate the validation of multiple-year patterns of vertebral growth—an often neglected component of elasmobranch aging studies (Beamish and McFarlane 1983; Brooks et al. 1994).

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